# Identification of a second site compensatory mutation in the Fe-protein that allows diazotrophic growth of *Azotobacter vinelandii* UW97

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**Abstract** ***Azotobacter vinelandii*** **UW97 is defective in nitrogen fixation due to a replacement of serine at position 44 by phenylalanine in the Fe-protein [Pulakat, L., Hausman, B.S., Lei, S. and Gavini, N. (1996) J. Biol. Chem. 271, 1884–1889]. Serine residue 44 is located in a conserved domain that links the nucleotide binding site and the MoFe-protein docking surface of the Fe-protein. Therefore, it is possible that the loss of function by *A. vinelandii* UW97-Fe-protein may be caused by global conformational disruption or disruption of the conformational change upon MgATP binding. To determine whether it is possible to generate a functional nitrogenase complex via a compensating second site mutation(s) in the Fe-protein, we have attempted to isolate genetic revertants of *A. vinelandii* UW97 that can grow on nitrogen-free medium. One such revertant, designated *A vinelandii* BG9, encoded a Fe-protein that retained the Ser44Phe mutation and also had a second mutation that caused the replacement of a lysine at position 170 by glutamic acid. Lysine 170 is highly conserved and is located in a conserved region of the Fe-protein. This region is implicated in stabilizing the MgATP-induced conformation of the Fe-protein and in docking to the MoFe-protein. Further complementation analysis showed that the Fe-protein mutant that retained serine 44 but contained the substitution of lysine at position 170 by glutamic acid was also non-functional. Thus, neither Ser44Phe nor Lys170Glu mutants of Fe-protein were functional; however, the Fe-protein in *A. vinelandii* BG9 that contained both substitutions could support diazotrophic growth on the strain.** 

*Key words:* *nifH*; Nitrogenase; Fe-protein; Second site mutation; *Azotobacter*

## 1. Introduction

The enzyme nitrogenase catalyzes the MgATP-dependent reduction of $N_2$ to ammonia. It is composed of two separately purified proteins, the Fe-protein and the MoFe-protein, encoded by *nifH* and *nifDK* genes, respectively [1,2]. The Fe-protein is a homodimer of 60 kDa in size containing a single [4Fe–4S] center, while the MoFe-protein is an $\alpha_2\beta_2$ tetramer of 240 kDa in size containing two pairs of metal centers, the P-clusters and FeMo-cofactors [3–8]. The Fe-protein functions as an electron donor to the MoFe-protein. This electron transfer is thermodynamically unfavorable and requires the hydrolysis of 2MgATP per electron transferred [9–13]. Apart from the critical role that the Fe-protein plays in electron transfer to the MoFe-protein in a MgATP-dependent reaction, the Fe-protein also has at least two additional functions. Firstly, it is involved in the initial biosynthesis of FeMo-cofactor [14–16]. Although details of the actual pathway for FeMo-cofactor biosynthesis have not yet been established, it has been documented that the FeMo-cofactor is synthesized separately from the MoFe-protein polypeptides [17,18]. At least seven *nif* genes – *nifB*, *nifE*, *nifH nifN*, *nifQ*, *nifV* and *nifX* – are known to be involved in this process [19–22]. The first evidence of the involvement of *nifH* in cofactor biosynthesis came from the observation that the mutant *Azotobacter vinelandii* DJ54, which does not synthesize the *nifH* polypeptide, does not synthesize the FeMo-cofactor [15]. Purification of the FeMo-cofactor-deficient MoFe-protein from this strain and its subsequent characterization by biochemical and spectroscopic analysis further confirmed the speculated role of NifH in cofactor biosynthesis [14]. Secondly, the Fe-protein's function also involves its participation in the maturation of an inactive FeMo-cofactor-deficient MoFe-protein [16,23,24]. Recently it was shown that assembly of the active MoFe-holoprotein requires MgATP and apodinitrogenase reductase (the Fe-protein without the [4Fe–4S] cluster) [25]. Even though the binding of MgATP to the apodinitrogenase reductase is required, MgATP hydrolysis is not needed for its ability to participate in the assembly and maturation of the MoFe-holoprotein [23,26].

Upon MgATP binding, five regions of the Fe-protein undergo detectable conformational changes [27,28]. Those regions are involved in nucleotide binding, ligating the [4Fe–4S] cluster, and forming the MoFe-protein docking surface of Fe-protein. Any change causing conformation disruption could cause the loss of the Fe-protein function by impairing MgATP-induced conformational changes, MgATP hydrolysis, complex formation, or complex dissociation [12,23,29–39]. However, the mutation of amino acids that are not located in the regions that are thought to be important to the conformational integrity of the Fe-protein functions could also result in a non-functional Fe-proteins [31,38]. Those residues are most likely associated with the regions involved in ATP hydrolysis or transducing the signal from nucleotide binding site to either the [4Fe–4S] cluster or the MoFe-protein docking site. As reported earlier, the *A. vinelandii* UW97 Fe-protein (Ser44Phe) lost its function in both FeMo-cofactor biosynthesis/insertion and electron transfer to the MoFe-protein [33,40]. Serine 44 is located in a peptide chain which links the nucleotide binding site and the MoFe-protein docking surface of the Fe-protein [4,27]. It has also been suggested that this region may participate in the MgATP-induced conformational changes of the Fe-protein, MgATP hydrolysis, and the dissociation of the Fe-protein-MoFe-protein complex [4,31]. How-

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ever, this region (Gly-37 through Thr-45) does not undergo dramatic conformational changes upon MgATP binding [27], indicating that the loss of function by Ser44Phe–Fe-protein may be caused by global conformational disruption or by the disruption of the conformational change upon MgATP binding. Therefore, mutations either at the docking surface of the Fe-protein or the docking sites of the MoFe-protein to fit Ser44Phe–Fe-protein could result in a functional nitrogenase complex. To isolate genetic revertants of *A. vinelandii* UW97 that can grow on nitrogen-free medium, cells were grown for several generations on $BN^+$ medium at 30°C and then plated on $Nif^-$ agar medium to score for forced revertants with the diazotrophic phenotype. This procedure resulted in the isolation of a revertant which exhibited a $Nif^+$ phenotype and showed diazotrophic growth similar to that of the *A. vinelandii* OP strain (Fig. 1). This strain was designated *A. vinelandii* BG9 (Table 1).

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strains were grown at 37°C in Luria broth or 2YT [41]. The ampicillin and tetracycline were used to a final concentration of 50 μg/ml and 20 μg/ml respectively wherever the selection was made. *A. vinelandii* strains were grown at 30°C in modified Burk's nitrogen-free ($BN^-$) medium [42]. When it was necessary to include fixed nitrogen in the medium, ammonium acetate ($NH_4OAc{\cdot}H_2O$) was added to a final concentration of 400 μg/ml.

### 2.2. Isolation of genetic revertant of A. vinelandii UW97

*A. vinelandii* UW97 was grown in 5 ml of $BN^+$ liquid medium until its $OD_{600}$ reached about 0.4–0.6. The cells were harvested by centrifugation at 4000 rpm, for 5 min. The cell pellets were washed three times by resuspending in 5 ml of $BN^-$ medium to remove traces of ammonia, and collecting the cells by centrifugation. At the last step the cells were resuspended in 1 ml of $BN^-$ medium and about 0.2 ml of this cell suspension was plated on $BN^-$ plates. The colonies were allowed to grow at 30°C. After 4 days of incubation, single colonies corresponding to spontaneous revertants appeared on nitrogen-free agar plates. One of these revertant colonies designated *A. vinelandii* BG9 (Table 1) was subjected to further analysis.

### 2.3. Construction of nifHp–lacZ in A. vinelandii BG9

To generate a nifHp–lacZ mutation on *A. vinelandii* BG9 chromosome, a Tn5-B21 mutagenesis was employed by a modified method described by Joerger et al. [43]. The plasmid pBG507 (Table 1) was introduced into *E. coli* strain ($supE^-$) and colonies carrying this plasmid were infected with λ:Tn5-B21 phage suspension as described previously [43]. The mixture was incubated for 2 h at 37°C. The infected cells were plated onto LB plate containing tetracycline (20 μg/ml) and allowed to grow for 24 h. $Tet^r$ cells were pooled and plasmid DNA was isolated. The plasmid DNA carrying Tn5-B21 in nifHp was designated pBG510. This plasmid was used to deliver nifHp:lacZ fusion onto the chromosome of *A. vinelandii* BG9 as described below.

### 2.4. General molecular biology techniques

PCR amplifications [44], DNA sub-cloning, plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations and *E. coli* transformations were carried out as described in the laboratory manuals [41] or as suggested in the manufacturers instructions. The purified PCR products were either used to transform *A. vinelandii* strains or cloned into pCR™ vector for DNA sequence analysis. Restriction enzymes were purchased either from Boehringer Mannheim (Indianapolis, IN, USA) or from Promega (Madison, WI, USA). Oligonucleotides used for PCR amplification were purchased from Gibco BRL Life Technologies Inc. (Gaithersburg, MD, USA). Radiolabeled material for sequencing ([$^{35}$S]dATP) was obtained from Dupont NEN (Boston, MA, USA). The nucleotide sequencing was performed using $T_7$ sequencing kit purchased from USB-Amersham Inc. (Cleveland, OH, USA).

### 2.5. Construction of A. vinelandii mutant strains

To construct *A. vinelandii* strains that contained site-directed mutations in the *nifH* gene, a gene replacement technique previously described in detail was used [45,46]. *A. vinelandii* strains have a very efficient recombination system that allows homologous recombination between the newly delivered sequence and the host chromosome. The plasmids used in the study are derivatives of pUC18 and pCR2.1 carrying the mutated version of the *nifH* gene. These non-replicative plasmids were used to transform *A. vinelandii* and the transformants were selected on $BN^-$ agar where ever the selection was made for $N_2$ fixation proficient strains.

## 3. Results and discussion

### 3.1. Localization and identification of a second site mutation in the Fe-protein (Ser44Phe) that restored the wild type Fe-protein function in A. vinelandii BG9

The *A. vinelandii* BG9 could acquire the $Nif^+$ phenotype by several different types of mutations. It may be that the mutation that is responsible for the $Nif^+$ phenotype of this strain is a simple replacement of phenylalanine at position 44 back to serine as in the wild type [33], another complementing substitution (unpublished data) or a second mutation located at a different position in the Fe-protein or in one of the subunits of the MoFe-protein. It is also possible that the second mutation is located in one of the accessory proteins of the *nif*-system [47] and has resulted in the modification of the functions of that accessory protein; or that it is located in the structural or accessory genes of the *vnf*- or *anf*-system [48] and has resulted in the activation of that system in the presence of molybdenum. Therefore, our first task was to determine if the mutation responsible for the $Nif^+$ phenotype of *A. vinelandii* BG9 was located in the *nif*-system or in one of the other two alternative systems. To do this we constructed a plasmid designated pBG510 in which the *nif*-structural genes were disrupted by insertion of *lacZ*-Tet cartridge [43] on the *nifH* gene. This was done by subjecting the *nifH* gene present on the plasmid

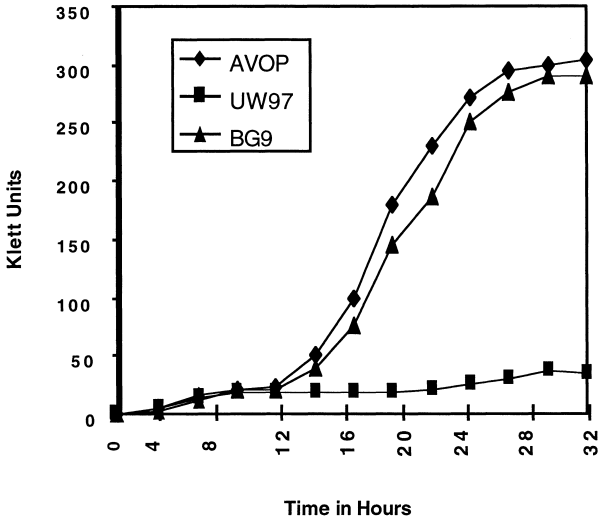


Fig. 1. Growth characteristics of *A. vinelandii* strains in $BN^-$ medium. An overnight culture was prepared by inoculating freshly growing colonies from agar plates into Burk's medium supplemented with ammonium acetate. Cells from the overnight culture were collected and washed with $BN^-$ medium and then transferred to side-arm flasks that contained the same medium. Cell densities were recorded over 32 h time periods. This analysis showed that the *A. vinelandii* BG9 strain has similar growth characteristics as that of the *A. vinelandii* OP.

Table 1
Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics and description | Source or reference |
|---|---|---|
| *E. coli* TG1 | K12, Δ(*lac-pro*), supE, *thi*-1, *lac*I$^q$, *lacZ*ΔM15, *hsd*D5 [F′ *traD36 proA*$^+$*B*$^+$] | Amersham life sciences Inc. IL, USA |
| *E. coli* HB101 | F$^-$ *sup*E44 *thi*-1 *hsd*S20 ($r_B$-, $m_B$-) *pro*A2 *ara*14 *gal*K *lac*Y1 *rsp*L20 (Str$^r$) *xyl*-5 *mtl*-1 *rec*A 13 *mcr*B *leu*B6 | Invitrogen, Carlsbad, CA, USA. |
| *A. vinelandii* OP | Wild type, Nif$^+$, soil bacterium. | Laboratory stock |
| *A. vinelandii* DJ54 | Nif$^-$; defined deletion in the *nifH* gene. | [14,15] |
| *A. vinelandii* UW97 | Nif$^-$; isolated by following nitrosoguanidine mutagenesis. The specific mutation responsible for the Nif$^-$ phenotype is the substitution of a non-conserved serine at position 44 of the Fe-protein by phenylalanine. | [33,40] |
| *A. vinelandii* BG9 | Nif$^+$; Spontaneous revertant of *A. vinelandii* UW97; this strain contained a functional *A. vinelandii* UW97-derived Fe-protein | This work |
| pCR®2.1 | Amp$^r$Kan$^r$; 3.9 kb used for cloning PCR products | Invitrogen, Carlsbad, CA, USA. |
| pBG507 | Derivative of pCR®2.1 in which a 940 bp DNA fragment that encodes the NifH open reading frame from *A. vinelandii* BG9 was cloned. This fragment was generated by PCR amplification using the primers complementary to the DNA immediately outside of NifH coding sequence. | This work |
| pBG508 | Derivative of pCR®2.1 in which a 940 bp DNA fragment that encodes the NifH open reading frame was cloned. The NifH open reading frame contained serine at position 44, which is same as in the wild type NifH and at position 170 a substitution of glutamic acid to replace lysine. | This work |
| pBG510 | Derivative of pUC18 in which a 940 bp DNA fragment that encodes the NifH open reading frame was cloned. Then the NifH open reading frame was subjected to Tn5 mutagenesis to generate this plasmid that contained *nifH::lacZ*-Tet. | This work |

pUC18 to Tn5-B21 mutagenesis [43] to integrate the *lacZ*-Tet cartridge on the coding sequence of the *nifH*. The plasmid DNA samples isolated from tetracycline-resistant *E. coli* colonies were subjected to restriction enzyme analysis to localize the position of *lacZ*-Tet cartridge integration. The plasmid that carried the *lacZ*-Tet cartridge in the coding sequence of the *nifH* isolated by this procedure was designated pBG510. *A. vinelandii* BG9 was transformed with this plasmid and the transformants were selected on BN$^+$ agar medium supplemented with tetracycline. Since the plasmid pBG510 has a *colE*1 origin of replication which does not support replication in *A. vinelandii* cells, we could obtain Tet$^r$ transformants by the integration of the *lacZ*-Tet cartridge into the *nifH* by double point, reciprocal recombination. Since the structural genes of the *nif*-system – *nifH*, *nifD* and *nifK* – are continuous and constitute a single operon on the *A. vinelandii* chromosome [3,47], disruption of the *nifH* gene by integration of the *lacZ*-Tet cartridge can also disrupt the expression of the *nifD* and *nifK*. If the compensating mutation(s) is (are) in any of *nif*-structural genes, the integration of *lacZ*-Tet cartridge should disrupt the synthesis of functional nitrogenase. We observed that all the Tet$^r$ transformants of *A. vinelandii* BG9 were also Nif$^-$ suggesting that the second mutation was located in the *nif*-system. Therefore, the diazotrophic growth observed in *A. vinelandii* BG9 is due to the expression of the *nif*-system and not the activation of the *vnf*- or *anf*-system. To further determine whether any mutations had occurred in the promoter region that may have resulted in elevated expression of the *nif*-structural genes, the β-galactosidase activity of the *A. vinelandii* BG9 was determined under repressed and derepressed conditions. Since the *lacZ* gene is under the transcriptional control of *nifH* promoter, the β-galactosidase activity of the *A. vinelandii* BG9 was taken as a measure of the *nifHDK* promoter activity. The β-galactosidase activity of *A. vinelandii* BG9 observed under repressed and derepressed conditions was comparable to the β-galactosidase activity detected from unaltered *nifH*p under similar growth conditions. These results suggest that no mutations resulting in an elevated level of expression of the *nif*-structural genes have occurred in *A. vinelandii* BG9.

To determine which of the *nif*-structural genes of *A. vinelandii* BG9 carried the compensating mutation responsible for Nif$^+$ phenotype, we PCR-amplified [44] the DNA fragments corresponding to the coding sequence of the *nifH*, *nifD* and *nifK* genes using appropriate primers and the chromosome of *A. vinelandii* BG9. The primers used for PCR amplifying the *nifH* were 5′-GATATCATGCGTCAATGCGCCATCTACGGC-3′ and 5′-GGATCCT CAGACTTCGGCGGTTTTGCCGACGATGG-3′; for *nifD* were 5′-GACCGGTATGTCG CGCGAAGAGGTTGAAT-3′ and 5′-TCAGGCGCTGGCGGCGACTTTCTCGGCGCCTT-3′; and for *nifK* were 5′-GAGCCAGCAAGTCGATAAAATCAAAGCCAG-3′ and 5′-AGCGTACCA GGTCGTGGTTGTAGTCGGT-3′. The DNA fragments encoding the *nifH*, *nifD* or *nifK* from *A. vinelandii* BG9 (generated by PCR amplification) were used to transform the *A. vinelandii* UW97 [45,46]. Since the DNA fragments encoding the *nif*-structural genes share homology with the chromosome it was expected that they would undergo recombination with their counterparts present on the chromosome [46]. If any of these DNA fragments carried the complementing mutation of *A. vinelandii* BG9 responsible for Nif$^+$ phenotype, that fragment will be able to complement

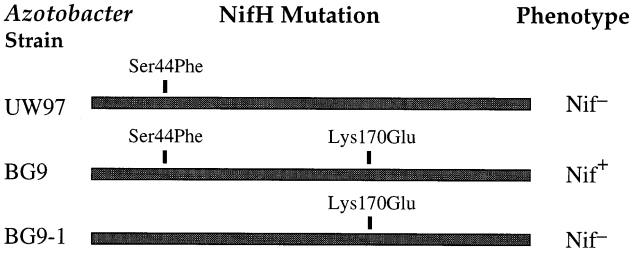


Fig. 2. Genetic map of the *nifH* gene and observed phenotype of *A. vinelandii* mutant strains are shown. The strains carrying single mutations (Ser44Phe, Lys170Glu) are Nif$^-$ whereas the strain carrying both mutations was able to show Nif$^+$ phenotype.

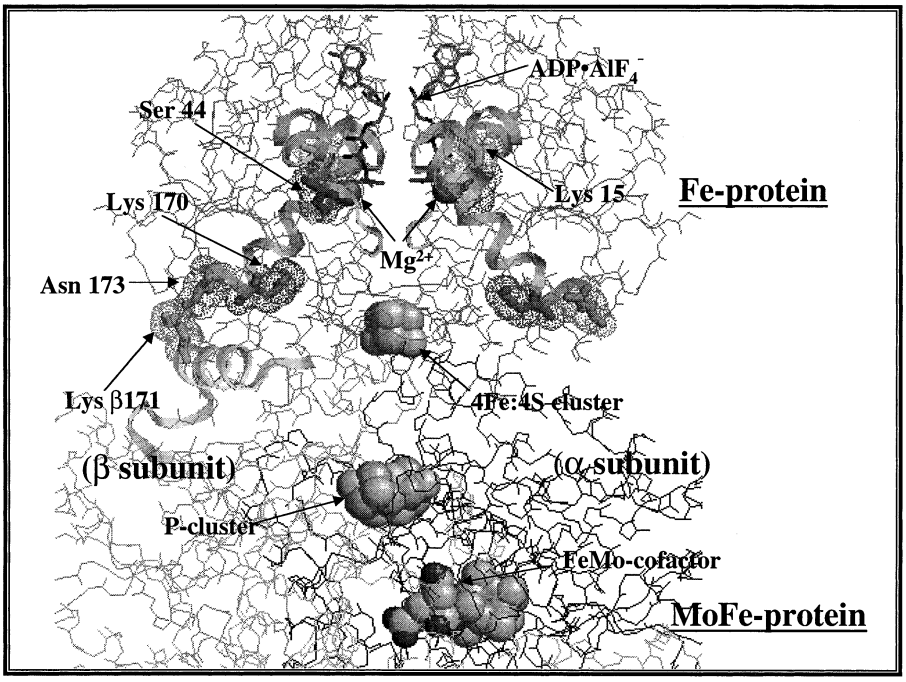


Fig. 3. A model showing the position of lysine 170 of Fe-protein based on the published structure of the complex between the Fe- and MoFe-proteins stabilized by MgADP·$AlF_4^-$. The lysine 170 which is changed to glutamic acid in the *A. vinelandii* BG9 strain is located very close to Asn-173 which interacts with lysine 171 of the β subunit of MoFe-protein. The serine 44 which was changed to phenylalanine in the Fe-protein of *A. vinelandii* UW97 strain is also marked.

the defect present in the nitrogenase of *A. vinelandii* UW97. Our results showed that the *A. vinelandii* UW97 cells transformed with the DNA fragment carrying the *nifH* coding sequence of *A. vinelandii* BG9 became $Nif^+$ (Fig. 2). On the other hand, the *A. vinelandii* UW97 cells transformed with DNA fragments that carried the *nifD* or *nifK* coding sequences of *A. vinelandii* BG9 remained $Nif^-$. These results suggested that the complementing mutation of *A. vinelandii* BG9 responsible for its $Nif^+$ phenotype is located in the *nifH* coding sequence.

To identify the exact location of the complementing mutation that rendered the *A. vinelandii* BG9 the $Nif^+$ phenotype, we cloned the DNA fragment carrying the *nifH* coding sequence of the *A. vinelandii* BG9 into pCR™2-1 (Table 1) and subjected it to nucleotide sequence analysis. This analysis showed that the original mutation, Ser44Phe, present in the Fe-protein of *A. vinelandii* UW97 was unaltered in Fe-protein of *A. vinelandii* BG9. Interestingly, we found another change in the *A. vinelandii* BG9 *nifH* coding sequence (Fig. 2). This second mutation was located at position 170 where a lysine codon (AAG) was replaced by a codon for glutamic acid (GAG). Therefore, substitution of lysine 170 by glutamic acid resulted in generating a functional UW97-derived Fe-protein. Thus it seems that in the *A. vinelandii* BG9 Fe-protein the defect due to Ser44Phe mutation was nullified by the second site mutation Lys170Glu.

To identify the contribution of the Lys170Glu which corrected the dysfunction caused by Ser44Phe mutation, we engineered a *nifH* gene that contained only the Lys170Glu mutation and this plasmid was designated pBG508. *A. vinelandii* UW97 and *A. vinelandii* DJ54 (Table 1) were transformed with pBG508 and the transformants were analyzed for an acquired $Nif^+$ phenotype. It was found that the pBG508 could not provide *A. vinelandii* UW97 or *A. vinelandii* DJ54 transformants with $Nif^+$ phenotype. The Ser44Phe mutation of Fe-protein resulted in a non-functional Fe-protein [33]; and the Lys170Glu mutation of the Fe-protein also resulted in a non-functional Fe-protein (Fig. 2). Taken together, these results indicated that the Ser44Phe and Lys170Glu double mutation in the *nifH* gene generated a new functional Fe-protein, although single mutations produced non-functional Fe-proteins.

### 3.2. Conclusions

Extensive mutational analysis combined with biochemical and spectroscopic methods has helped to determine the structure–function relationship of some of the conserved amino acid clusters in the Fe-protein [1,26]. From the crystal structure it is predicted that in the *A. vinelandii* Fe-protein functional salt bridges may form between Lys-15 and Asp-125. Substituting glutamine or proline for Lys-15 has shown that this lysine appears to be essential for the MgATP-induced conformational change of the wild type Fe-protein. Replacing Asp-125 with either glutamate (which has a similar charge) or with asparagine resulted in a $Nif^-$ phenotype, caused by its inability to undergo the MgATP-induced conformational change [39]. Another amino acid that is essential for the MgATP-induced conformational change of the wild type Fe-protein is alanine at position 157 [23,49].

Serine 44 and other residues of the conserved second domain are located at the core of the Fe-protein and around the MgATP binding site (Fig. 3). Asp-39 and Asp-43 are implicated in playing a role in the hydrolysis of MgATP. Ser-44 lies in between Lys-15 and Asp-125 (Fig. 3); therefore, the replacement of serine by phenylalanine would introduce a hydrophobic ring instead of the hydroxyl group at the side chain and might distort or even disrupt the salt bridge between Lys-15 and Asp-125. In addition, the side chain of Ser-44 is likely

to hydrogen bond to the nucleotide [4,39]; therefore, the substitution of serine at position 44 by phenylalanine may displace the nucleotide and cause other conformational shifts. Combining these effects, it can be assumed that the mutation Ser44Phe disrupts the structural rearrangement upon MgATP binding which is required for the docking of the Fe-protein onto the MoFe-protein. On the other hand, the highly conserved lysine 170 is located in the region of Cys-151 to Ser-176, which overlaps the highly conserved fifth domain of the Fe-protein (Fig. 3). The Met-156 located in this region of the Fe-protein, is suggested to be involved in interacting with Asp-43 and the nucleotide in the other subunit. Replacing this residue with cysteine induced an altered, non-productive conformational change to the Fe-protein when MgATP was bound [30]. Similarly, Ala157Ser–Fe-protein also was unable to undergo MgATP-induced conformational change [23,49]. It was also suggested that Ala-157 is involved in stabilizing initial conformational changes of helix α5 in the Fe-protein upon Mg-ATP binding [29]. Thus it seems that amino acids in this domain are essential for stabilizing the Mg-ATP induced conformational changes. Moreover, Lys-170 is very close to Asn-173 which interacts with Lys-171 of the β subunit of MoFe-protein in the nitrogenase complex (Fig. 3). The change from lysine to glutamic acid at position 170 would produce a much stronger interaction between Lys-171 of the β subunit of the MoFe-protein and Glu-170 and Asn-173 residues from the Fe-protein. This interaction or the local conformational changes caused by the substitution of lysine 170 by glutamic acid could let the Fe-protein and the MoFe-protein form a functional complex.

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